

## Pharmacodynamics of Polymyxin B against *Pseudomonas aeruginosa*

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Received 30 March 2005/Returned for modification 25 May 2005/Accepted 25 June 2005

Despite limited data, polymyxin B (PB) is increasingly used clinically as the last therapeutic option for multidrug-resistant (MDR) gram-negative bacterial infections. We examined the in vitro pharmacodynamics of PB against four strains of *Pseudomonas aeruginosa*. Clonal relatedness of the strains was assessed by random amplification of polymorphic DNA. Time-kill studies over 24 h were performed with approximately  $10^5$  and  $10^7$  CFU/ml of bacteria, using PB at 0, 0.25, 0.5, 1, 2, 4, 8, and  $16\times$  MIC. Dose fractionation studies were performed using an in vitro hollow-fiber infection model (HFIM) against a wild-type and a MDR strain. Approximately  $10^5$  CFU/ml of bacteria were exposed to placebo and three regimens (every 8 h [q8h], q12h, and q24h) simulating the steady-state unbound PB pharmacokinetics resulting from a daily dose of 2.5 mg/kg of body weight and 20 mg/kg (8 times the clinical dose). Samples were obtained over 4 days to quantify PB concentrations, total bacterial population, and subpopulation with reduced PB susceptibility ( $>3\times$  MIC). The bactericidal activity of PB was concentration dependent, but killing was significantly reduced with a high inoculum. In HFIM studies, a significant reduction in bacterial load was seen at 4 h in all active regimens, but selective amplification of the resistant subpopulation(s) was apparent at 24 h with the clinical dose (both strains). Regrowth was eventually observed in all dosing regimens with the MDR strain, but its occurrence was prevented in the wild-type strain by using 8 times the clinical dose (regardless of dosing intervals). Our results suggested that the bactericidal activity of PB was concentration dependent and appeared to be related to the ratio of the area under the concentration-time curve to the MIC.

The prevalence of antimicrobial resistance in gram-negative bacteria in hospitals is an increasing problem. *Pseudomonas aeruginosa* and *Acinetobacter* spp. are often implicated, and they are associated with significant morbidity and mortality. Multidrug resistance among these pathogens is especially worrisome, as the number of possible therapeutic options is severely limited. In a recent national surveillance of antimicrobial resistance in *P. aeruginosa* isolates obtained from intensive care units, the prevalence rate of multidrug resistance (defined as resistance to three or more of the following drugs: ceftazidime, ciprofloxacin, tobramycin, and imipenem) increased from 4% in 1993 to 14% in 2002 (20). Recently, there is much rekindled interest in using the polymyxins (polymyxin B and colistin) for the treatment of infections caused by multidrug-resistant (MDR) gram-negative organisms (6, 9, 11, 13, 15, 16, 23).

The polymyxins are polypeptide antibiotics isolated from *Bacillus polymyxa*, first made available for clinical use in the late 1950s and early 1960s. The polymyxins exert their bactericidal activity by binding to the bacterial cell membrane and disrupting its permeability, resulting in leakage of intracellular components. They also have antiendotoxin activity (2, 17). These agents are rapidly bactericidal against many gram-negative bacteria. Soon after their introduction into clinical use,

concerns arose about adverse effects (e.g., nephrotoxicity, ototoxicity, and neuromuscular blockade) associated with their use. As antimicrobial agents with better safety profiles became available, the clinical use of the polymyxins was quickly abandoned due to perceived toxic side effects (5, 10).

As a consequence of the increasing rates of multidrug resistance in gram-negative bacteria, the polymyxins have increasingly become the last viable therapeutic option for MDR pseudomonal infections, despite very limited pharmacokinetic and pharmacodynamic data (5, 10). As we are faced with the possibility of returning to the preantibiotic era, the polymyxins are the agents of our last line of defense. It is therefore critical that they be used judiciously and optimally. If the pharmacodynamics of these agents are thoroughly understood, dosing regimens may be designed rationally to optimize patient outcomes and to minimize the emergence of resistance to these agents (4). The objectives of this study were to evaluate the in vitro pharmacodynamics of polymyxin B against *P. aeruginosa* with respect to its bactericidal activity and propensity to suppress spontaneous (non-plasmid-mediated) emergence of resistance.

(This study was presented in part at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D.C., 30 October to 2 November 2004.)

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### MATERIALS AND METHODS

**Antimicrobial agent.** Polymyxin B sulfate powder (USP) was purchased from Sigma (St. Louis, MO). A stock solution at 1,024 mg/liter in sterile water was prepared, aliquoted, and stored at  $-70^{\circ}\text{C}$ . Prior to each susceptibility testing, an

aliquot of the drug was thawed and diluted to the desired concentrations with cation-adjusted Mueller-Hinton II broth (Ca-MHB) (BBL, Sparks, MD).

**Microorganism.** Four strains of *P. aeruginosa* were used in the study. A standard wild-type strain, ATCC 27853 (American Type Culture Collection, Rockville, MD), and three carbapenem-resistant clinical bloodstream isolates were examined. The bacteria were stored at  $-70^{\circ}\text{C}$  in Protect (Key Scientific Products, Round Rock, TX) storage vials. Fresh isolates were subcultured twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA) for 24 h at  $35^{\circ}\text{C}$  prior to each experiment. The clonal relatedness of the bacterial strains was assessed using rapid amplification of polymorphic DNA, using primer 208 (5'-ACGGCC GACC-3') as described previously (14).

**Susceptibility studies.** Polymyxin B MICs were determined for different strains of *P. aeruginosa* in Ca-MHB using a broth macrodilution method as previously described (18). The final concentration of bacteria in each broth macrodilution tube was approximately  $5 \times 10^5$  CFU/ml of Ca-MHB. Serial twofold dilutions of drug were used. The MIC was defined as the lowest concentration of drug that resulted in no visible growth after 24 h of incubation at  $35^{\circ}\text{C}$  in ambient air. Susceptibility to a screening panel of antimicrobial agents (consisting of piperacillin, ceftazidime, aztreonam, imipenem, meropenem, levofloxacin, and tobramycin) was determined by E-test (AB Biodisk, Piscataway, NJ) according to the manufacturer's instructions. The studies were conducted in duplicate and repeated at least once on a separate day.

**Time-kill studies.** All four bacterial strains were examined. Time-kill studies were conducted with different and escalating concentrations of polymyxin B. Seven clinically achievable concentrations of polymyxin B were used: 0 (control), 0.25, 0.5, 1, 2, 4, 8, and 16 mg/liter (5). An overnight culture of the isolate was diluted 30-fold with prewarmed Ca-MHB and incubated further at  $35^{\circ}\text{C}$  until reaching late-log-phase growth. The bacterial suspension was diluted with Ca-MHB accordingly based on absorbance at 630 nm; 15 ml of the suspension was transferred to 50-ml sterile conical flasks, each containing 1 ml of a drug solution at 16 times the target concentration. The final concentrations of the bacterial suspension in each flask at baseline were approximately  $1 \times 10^5$  CFU/ml and  $1 \times 10^7$  CFU/ml. In addition to the standard inoculum ( $1 \times 10^5$  CFU/ml), a high inoculum ( $1 \times 10^7$  CFU/ml) was also used to simulate the bacterial load in a severe infection. Furthermore, the high inoculum used would allow resistant subpopulations to likely be present at baseline. The experiment was conducted for 24 h in a shaker water bath set at  $35^{\circ}\text{C}$ . Serial samples (baseline and 2, 4, 8, 12, and 24 h) were obtained from each flask over 24 h to characterize the effect of various drug exposures on the total bacterial population. Prior to culturing the bacteria quantitatively, the bacterial samples (0.5 ml) were centrifuged at  $10,000 \times g$  for 15 min and reconstituted with sterile normal saline to their original volumes in order to minimize drug carryover effect. Total bacterial populations were quantified by spiral plating of  $10\times$  serial dilutions of the samples (50  $\mu\text{l}$ ) onto cation-adjusted Mueller-Hinton agar (MHA) plates (Hardy Diagnostics, Santa Maria, CA). The medium plates were incubated in a humidified incubator ( $35^{\circ}\text{C}$ ) for 18 to 24 h, and the bacterial density from each sample was determined with a CASBA-4 colony scanner and software (Spiral Biotech, Bethesda, MD). The theoretical lower limit of detection was 400 CFU/ml. Each experiment was repeated at least once on a separate day.

**Hollow-fiber infection model.** The schematic of the hollow-fiber infection model system has been described previously (1). Drug was directly injected into the central reservoir to achieve the peak concentration desired. Fresh (drug-free) growth medium was infused continuously from the diluent reservoir into the central reservoir to dilute the drug, in order to simulate drug elimination in humans. An equal volume of drug-containing medium was removed from the central reservoir concurrently to maintain an isovolumetric system. Bacteria were inoculated into the extracapillary compartment of the hollow-fiber cartridge (Fibercell Systems, Inc., Frederick, MD); the bacteria were confined in the extracapillary compartment but were exposed to the fluctuating drug concentration in the central reservoir by means of an internal circulatory pump in the bioreactor loop. The optional absorption compartment of the system was not used.

**Experimental setup.** Two strains (a wild-type strain, PA 27853, and an MDR clinical strain, PA 37) of *P. aeruginosa* were used. The inocula were prepared as described above. The bacteria (15 ml) were inoculated into the hollow-fiber infection models at a concentration of approximately  $1 \times 10^5$  CFU/ml. Dose fractionation studies were conducted for 96 h in a humidified incubator set at  $35^{\circ}\text{C}$ . The bacteria were exposed to placebo and 3 dosing regimens (every 8 h [q8h], q12h, and q24h), simulating the steady-state pharmacokinetic profiles of unbound polymyxin B (terminal half-life = 6 h) resulting from a daily dose of 2.5 mg/kg of body weight (standard clinical dose) and 20 mg/kg (8 times the standard clinical dose), respectively (Fig. 1A) (5).

**Pharmacokinetic validation.** Serial samples were obtained from the infection models on day 0 and day 2. Polymyxin B concentrations in these samples were assayed by a validated bioassay method as described below. The concentration-time profiles were modeled by fitting a one-compartment linear model to the observations, using the ADAPT II program (3). An unweighted (least-squares) error model structure was used.

**Bioassay.** Polymyxin B concentrations were determined by a microbioassay utilizing *Klebsiella pneumoniae* ATCC 13883 as the reference organism. The bacteria were incorporated into 30 ml of molten cation-adjusted MHA (at  $50^{\circ}\text{C}$ ) to achieve a final concentration of approximately  $1 \times 10^5$  CFU/ml. The agar was allowed to solidify in 150-mm medium plates. A size 3 cork bore was used to create nine wells in the agar per plate. Standards and samples were tested in duplicate with 40  $\mu\text{l}$  of the appropriate solution in each well on the same day. The polymyxin B standard solutions ranged from 4 to 256 mg/liter in Ca-MHB. The medium plates were incubated at  $35^{\circ}\text{C}$  for 24 h, and the zones of inhibition were measured. The assay was linear (correlation coefficient  $\geq 0.985$ ) using zone diameter versus log of the standard drug concentration. The intraday and interday coefficients of variation for all standards were  $<5\%$  and  $<6\%$ , respectively.

**Microbiologic response.** Serial samples (0.5 ml) were also obtained at baseline, at 4 h, at 8 h (on day 0), and daily (before first dose of the day) in duplicate from each hollow-fiber system, for quantitative culture to define the effects of various drug exposures on the total bacterial population and on selection of resistant bacterial subpopulations. Total bacterial populations were quantified as described above. Subpopulations with reduced susceptibility (resistant) were quantified by culturing onto MHA plates supplemented with polymyxin B at  $3\times$  MIC. Since susceptibility testing is performed in twofold dilutions and a one-tube difference (one doubling in concentration) is commonly accepted as reasonable interday variation, quantitative cultures on drug-supplemented medium plates (at  $3\times$  MIC) would allow reliable detection of bacterial subpopulations with reduced susceptibility. The medium plates were incubated at  $35^{\circ}\text{C}$  for up to 24 h (total population) and 72 h (subpopulations with reduced susceptibility); bacterial density from each sample was estimated as described above.

**Studies on polymyxin B-resistant isolates.** Bacterial isolates were recovered from polymyxin B-supplemented plates at the end of the experiment, and polymyxin B susceptibility testing was repeated to confirm the presence of resistance (to rule out degradation of drug supplementation in MHA, giving rise to falsely resistant isolates). To provide insights on the mechanism of polymyxin B resistance and cross-resistance to other agents, susceptibility testing of the resistant isolates (obtained from the wild-type parent) was also repeated using a screening panel of antimicrobial agents (consisting of piperacillin, ceftazidime, aztreonam, imipenem, meropenem, levofloxacin, tobramycin, and colistin). To further elucidate if polymyxin B resistance was adaptive (reversible) or stable (nonreversible), three resistant isolates obtained from the wild-type parent (one from each dosing schedule) were serially passaged daily on drug-free 5% blood plates. Tests for susceptibility to polymyxin B and colistin were repeated on days 5, 10, and 20.

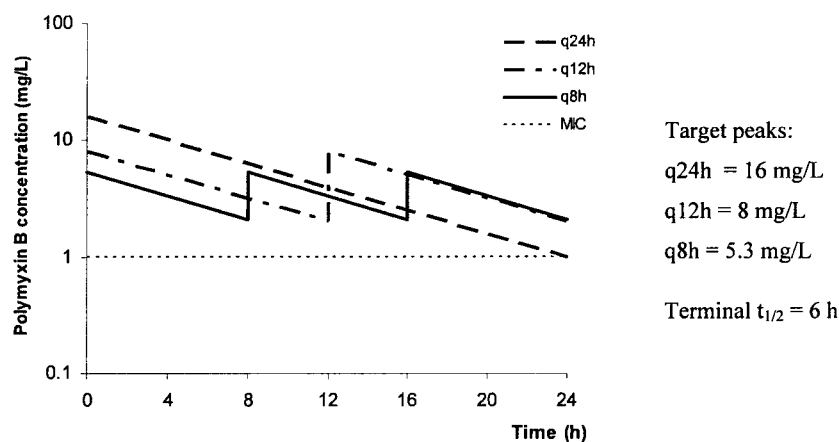
## RESULTS

**Microorganism and susceptibility studies.** The bacterial strains were found to be clonally unrelated (data not shown), and their susceptibilities were as shown in Table 1. The polymyxin B MIC ranged from 0.5 to 1 mg/liter.

**Time-kill studies.** Very similar killing profiles were observed in all bacterial strains. Rapid and significant declines ( $>2$ -log drop) in bacterial burden were observed after 2 h in all drug exposures. The bactericidal activity of polymyxin B was found to be concentration dependent. With increasing concentrations of polymyxin B, a higher killing rate and a greater extent of killing were seen with the standard inoculum of  $10^5$  CFU/ml, as shown in Fig. 2. A similar trend was also observed with the higher inoculum ( $10^7$  CFU/ml), but killing was significantly reduced, most likely due to the inoculum effect (Fig. 3). Regrowth was evident after the initial reduction in bacterial burden in all time-kill studies.

**Pharmacokinetic validation in hollow-fiber infection models.** All simulated polymyxin B exposures were satisfactory; a typical simulated pharmacokinetic profile (20 mg/kg/day q12h) was as shown in Fig. 1B.

A



B

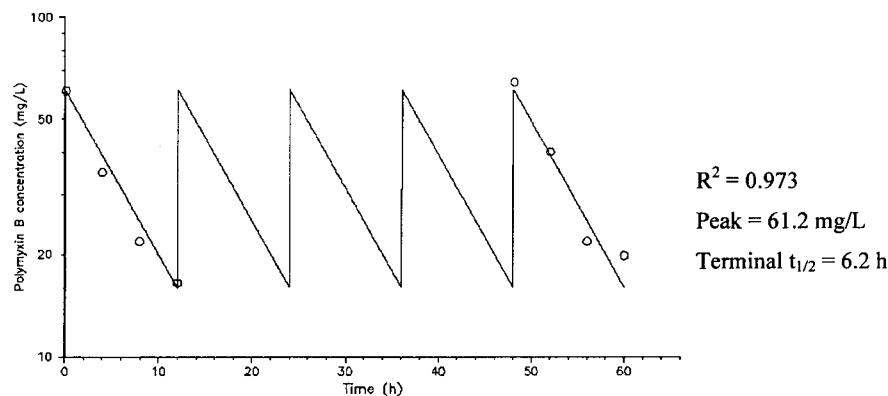


FIG. 1. Simulated polymyxin B pharmacokinetic profiles. (A) Target for daily dose of 2.5 mg/kg; (B) observed pharmacokinetic profile with 20 mg/kg/day given every 12 h.

**Microbiologic response.** With the simulated standard dose (2.5 mg/kg/day), all active regimens showed a significant killing of both strains of bacteria at 4 and 8 h. However, regrowth was apparent with repeated dosing beyond 24 h (Fig. 4), similar to that observed in time-kill studies. Regrowth observed over time was likely due to amplification of resistant populations, as demonstrated in Fig. 5. Susceptible bacterial populations were selectively eradicated, resulting in unopposed growth of resis-

tant subpopulations and consequently the emergence of resistance over time. As long as the total daily dose (exposure) remained the same, dosing schedules appeared to have little impact on the bactericidal activity of polymyxin B.

A higher dose (20 mg/kg/day, 8 times the clinical dose) was simulated to examine if resistance in *P. aeruginosa* could be counterselected. A sustained reduction in total bacterial burden and suppression of the resistant subpopulation were achieved over 96 h for the wild-type isolate (Fig. 6) but not for the MDR isolate (data not shown [similar to those in Fig. 4B]). The observed difference may be due to the difference in baseline mutation frequency between the two isolates. Despite the low inoculum used in the hollow-fiber infection model, we found an unexpectedly high mutation frequency (approximately 1 in  $10^3$  CFU) of the MDR strain at  $>3 \times$  MIC (approximately 100 times that in the wild-type strain). As noted previously, dosing schedules did not have an impact on the propensity of polymyxin B in suppressing resistance emergence.

**Studies on polymyxin B-resistant isolates.** The resistant isolates were found to have an 8- to 16-fold increase in MIC of

TABLE 1. Susceptibilities of bacterial isolates used

Strain	MIC (mg/liter) of <sup>a</sup> :								
	PB	PIP	CAZ	ATM	IPM	MEM	LVX	TOB	COL
PA 27853	1	1.5	1	2	3	0.38	0.75	0.5	1.5
PA 3	0.5	6	3	8	<b>&gt;32</b>	<b>&gt;32</b>	<b>&gt;32</b>	1	ND
PA 5	1	6	2	8	<b>&gt;32</b>	<b>&gt;32</b>	2	0.38	ND
PA 37	1	<b>&gt;256</b>	3	16	<b>&gt;32</b>	<b>&gt;32</b>	<b>&gt;32</b>	<b>384</b>	ND

<sup>a</sup> PB, polymyxin B; PIP, piperacillin; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; LVX, levofloxacin; TOB, tobramycin; COL, colistin (polymyxin E); ND, not determined. Bold type indicates resistant phenotypes based on NCCLS guidelines.

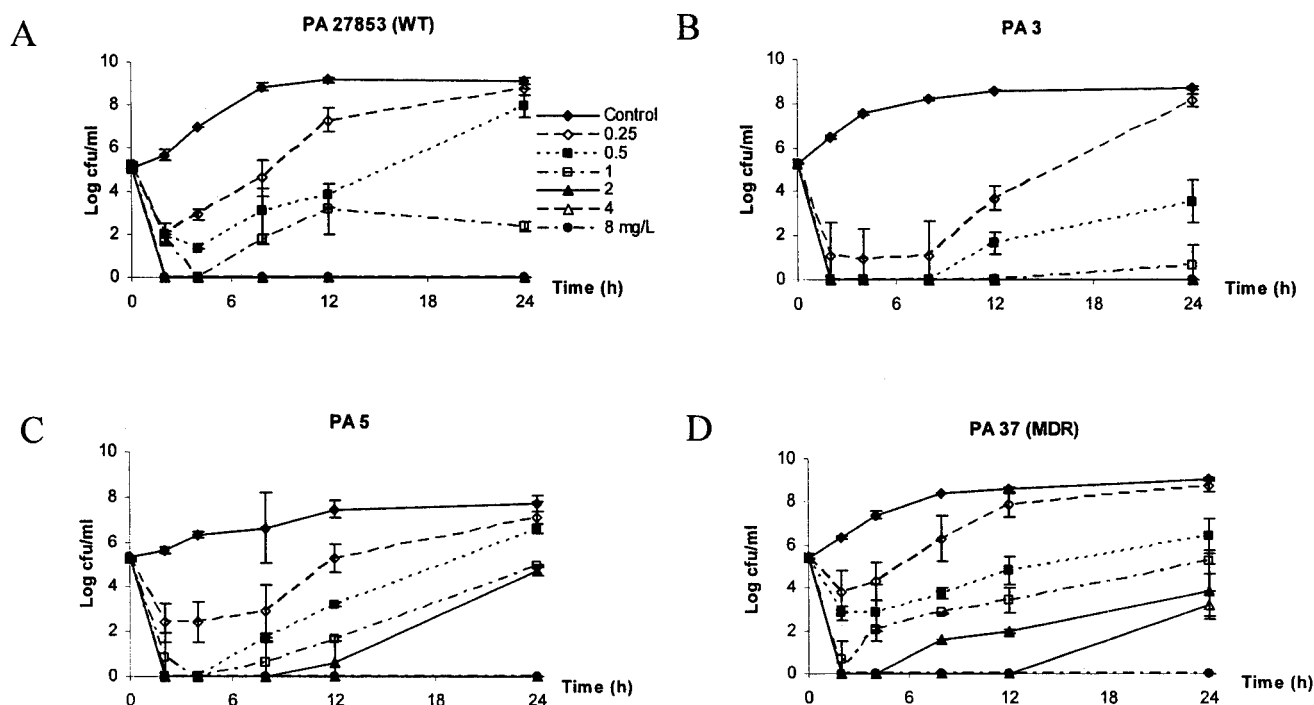


FIG. 2. Time-kill studies of polymyxin B with the standard inoculum ( $10^5$  CFU/ml). Data are presented as means and standard deviations. WT, wild type.

polymyxin B, compared to their parent strains. Cross-resistance to other antimicrobial agents in the screening panel was not observed, except for colistin (a 4- to 16-fold increase compared to wild-type parent strain). Upon serial passage on drug-free medium plates over 20 days, susceptibility reversal (to both polymyxin B and colistin) was observed in one of the three isolates investigated, suggesting that resistance to the polymyxins might be adaptive (nonmutational).

## DISCUSSION

With the alarming increase in multidrug resistance in gram-negative bacteria, many antimicrobial agents are being rendered ineffective. The polymyxins (polymyxin B and colistin) are increasingly used clinically as our last viable therapeutic option. To date, clinical experience with polymyxin B is still very limited (21, 23; A. L. H. Kwa, P. L. Choo, A. Tan, J. Low, and B. H. Tan, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. K-701, p. 360, 2003), and relatively little is known about its efficacy in treating severe infections.

Most investigations on the pharmacodynamics of the polymyxins have focused on colistin (polymyxin E) so far (8, 12), and less is known about the pharmacodynamics of polymyxin B. Improved understanding of the pharmacodynamics of polymyxin B may help to design dosing regimens rationally in order to optimize patient outcomes and retard the emergence of resistance (4). To the best of our knowledge, this is the first study examining the pharmacodynamics of polymyxin B. In addition to time-kill studies, we also employed an in vitro hollow-fiber infection model to explore the bactericidal activity of clinically relevant (achievable) polymyxin B exposures

against *P. aeruginosa*. Furthermore, the feasibility of optimizing the pharmacodynamics of polymyxin B in suppressing the emergence of resistance was also examined.

Consistent with previous findings on colistin, we found that the killing of *P. aeruginosa* by polymyxin B in time-kill studies was concentration dependent (8, 12). As with colistin, initial killing was rapid, but regrowth was readily seen in all time-kill studies. In addition, the killing of *P. aeruginosa* was reduced if a higher inoculum was used at baseline, suggesting that polymyxin B might be susceptible to the inoculum effect. The explanation of regrowth was not specifically investigated, but it appeared that the killing activity of polymyxin B was not sustained.

We further explored the potential clinical utility of various concentration-time profiles of polymyxin B in a hollow-fiber infection model. We used a dose fractionation study design (same daily dose but various doses and different dosing intervals used) to delineate which pharmacodynamic parameter (e.g., ratio of area under the concentration-time curve to MIC [AUC/MIC], ratio of maximum concentration of drug in serum to MIC, percentage of the dosing interval that the drug concentration was above the MIC, etc.) was most closely linked to the bactericidal effect of polymyxin B. Our experimental data consistently revealed that altering the dosing schedule (with identical daily dose) did not appear to have influenced the killing or resistance suppression against two strains of *P. aeruginosa*, suggesting that the pharmacodynamics of polymyxin B was most closely linked to the AUC/MIC ratio.

Despite the fact that rapid and substantial initial killing was observed, the standard clinical dosing resulted in regrowth and resistance emergence over 4 days in both the wild-type and

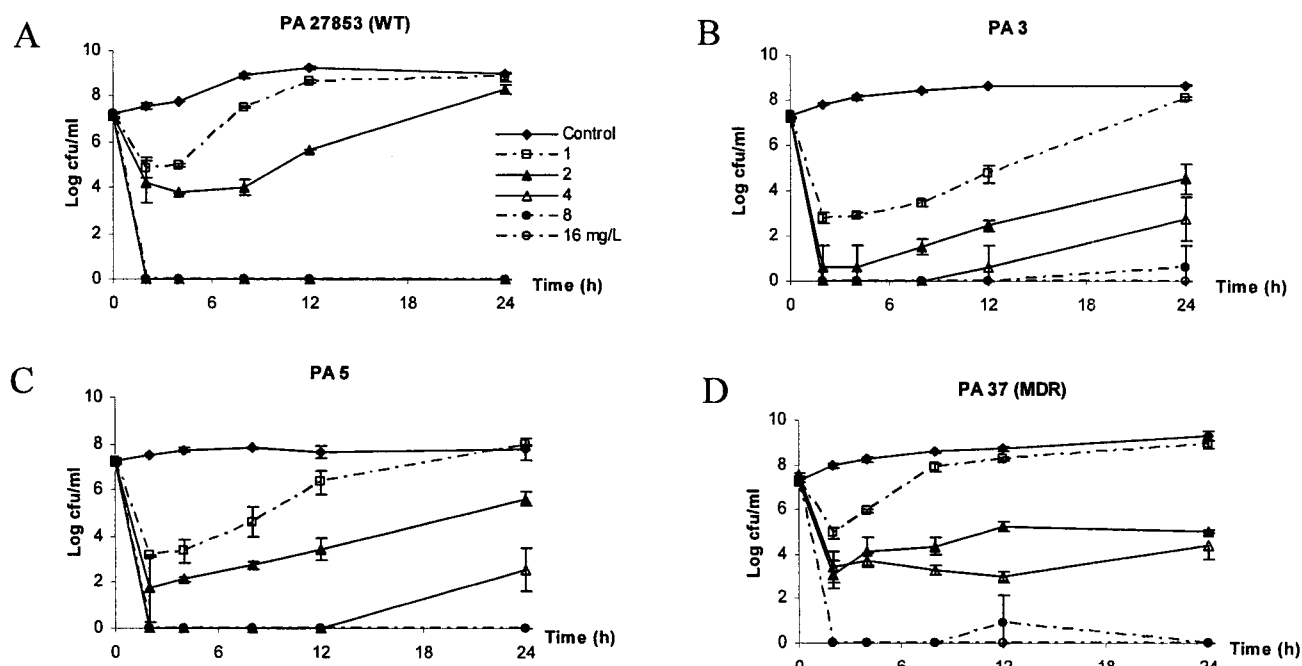


FIG. 3. Time-kill studies of polymyxin B with a higher inoculum ( $10^7$  CFU/ml). Data are presented as means and standard deviations. WT, wild type.

MDR bacterial strains. By validating our simulated pharmacokinetic exposures and using polymyxin B-supplemented medium plates for quantitative cultures, we demonstrated that regrowth was likely due to adaptation and/or selective amplification of resistant subpopulations (as opposed to degradation of polymyxin B over time). This unexpected result cautions against the standard polymyxin B daily dose of 2.5 mg/kg as monotherapy in an immunocompromised host, as it may not be adequate for the treatment of infections caused by *P. aeruginosa*. Clinical experience with this cohort is limited. However, data obtained with immunocompetent patients suggested that polymyxin B therapy was reasonably efficacious against infections caused by MDR gram-negative organisms, if used in combination with other agents (21, 23).

The mechanism of resistance to the polymyxins remains poorly understood. It is believed to be due to loss of lipopolysaccharide (7) or replacement of magnesium by protein H1 in

the outer membrane (19, 22). In this study, we did not investigate the specific mechanism of polymyxin B resistance. However, cross-resistance between the polymyxins (polymyxin B and colistin) was demonstrated, but not cross-resistance with any of the  $\beta$ -lactams, quinolone, and aminoglycoside investigated. More interestingly, there might be more than one type of non-plasmid-mediated resistance mechanism, as suggested previously (5). One of the polymyxin B resistance mechanisms appeared to be stable (mutational), while the other might be reversible upon removal of selective pressure (adaptive). While mutational resistance occurs infrequently (more commonly seen with an inoculum size greater than the mutation frequency of resistance), adaptive resistance may occur more readily (even with a standard inoculum size). This finding is consistent with our observations in time-kill studies, in which regrowth occurred readily (within 12 h) after the initial decline in bacterial burden. In addition, in the hollow-fiber infection

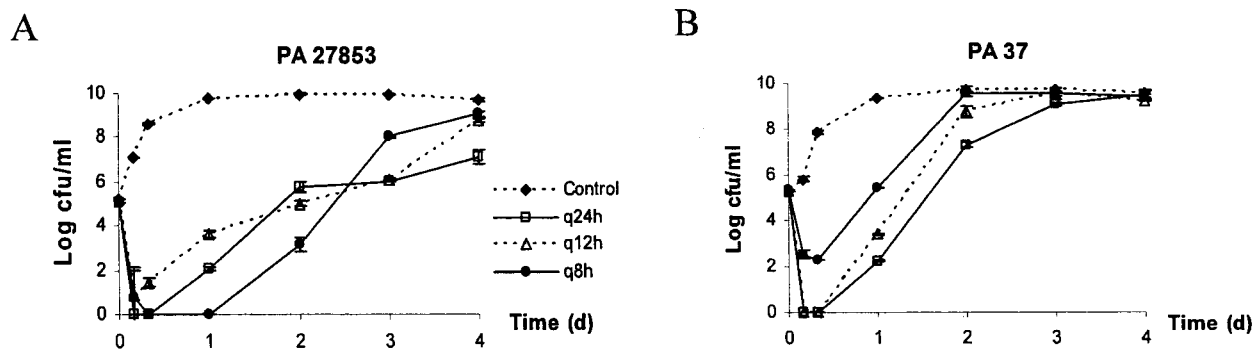


FIG. 4. Biologic response observed in hollow-fiber infection models with standard dose (2.5 mg/kg/day).

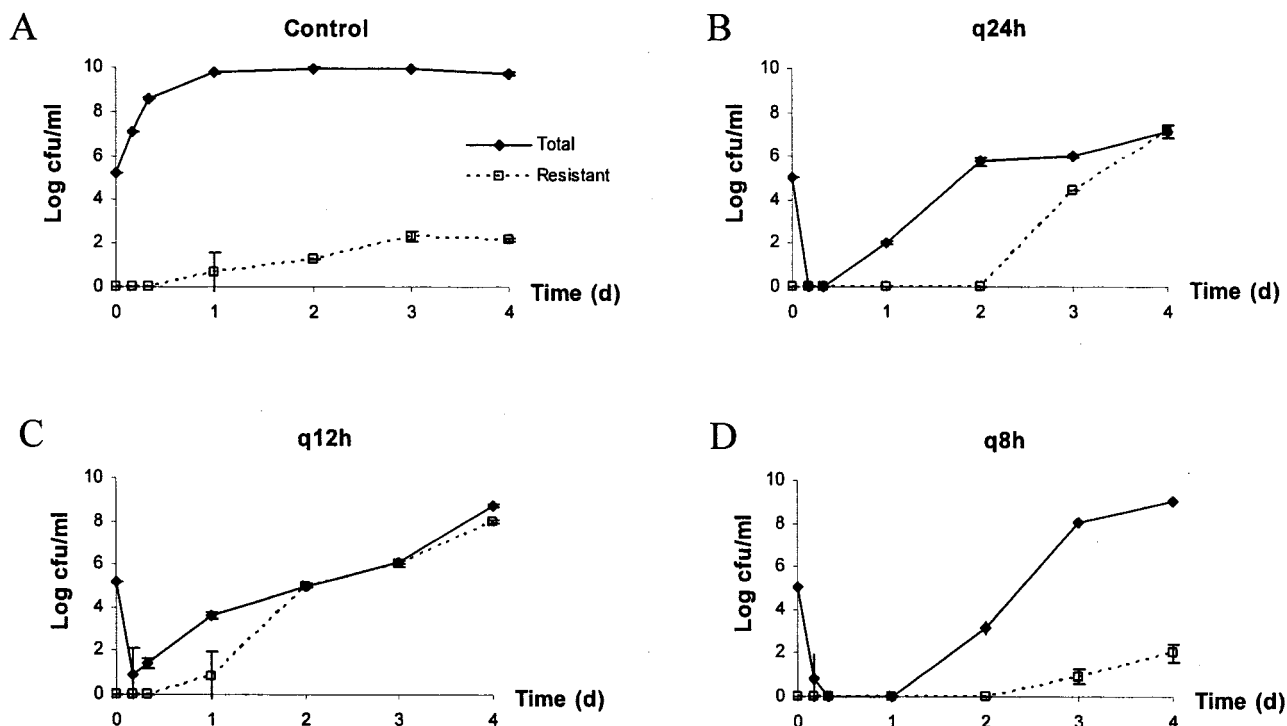


FIG. 5. Selective amplification of the resistant subpopulation observed with standard dose (2.5 mg/kg/day) in PA 27853.

models, repeated (standard daily) dosing did not result in a sustained suppression of the bacterial burden.

We further explored the feasibility of using elevated drug exposure to counterselect resistance. We noted that polymyxin B-resistant isolates had an 8- to 16-fold increase in MIC, compared to their parent strain. Therefore, we investigated the effect of 8 times the standard clinical dose on the time courses of the bacteria. Using 8 times the standard clinical dose, all simulated dosing regimens would have a concentration of  $\geq 8 \times$  MIC of the parent strain throughout the entire dosing interval (time above  $8 \times$  MIC, 100%). We found that the emergence of resistance could be suppressed using this simulated daily dose for the wild-type strain, somewhat consistent with our postulation of regrowth due to adaptive resistance. However, we were still unable to prevent the emergence of resistance in the MDR strain, despite using 8 times the standard clinical dose.

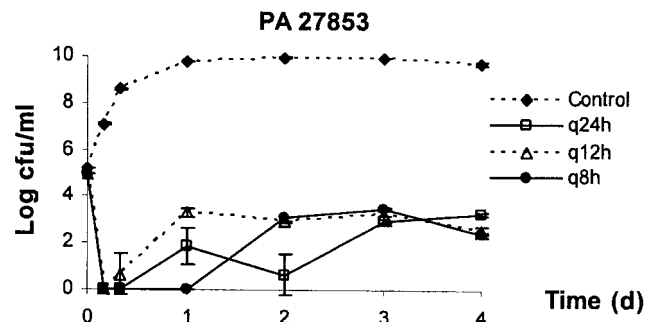


FIG. 6. Biologic response observed in hollow-fiber infection models with 8 times the standard dose (20 mg/kg/day).

This observation is consistent with those observed in time-kill studies, in which higher polymyxin B concentrations were necessary to achieve the same bacterial burden reduction in the MDR strain. However, given that the time-kill studies were conducted for only 24 h, there might not have been enough time for the resistant subpopulation to proliferate and dominate the entire bacterial population. On the other hand, the hollow-fiber infection model studies were conducted over 96 h. The resistant subpopulations were selectively amplified, resulting in regrowth over time despite using a higher daily dose. We noted that the baseline mutation frequency of the MDR strain to polymyxin B (at  $3 \times$  MIC) was approximately 100 times higher than that observed in the wild type. In spite of an apparently low MIC, this strain was likely to be hypermutable and much more difficult to suppress, as reflected in our time-kill studies (Fig. 2D and 3D). Consequently, regrowth was likely due to a combination of both adaptive resistance and selective amplification of mutational resistance. Combination therapy may be considered for treatment of infections caused by this strain.

**Conclusion.** Our results suggested that polymyxin B exhibits rapid and concentration-dependent bactericidal activity against *P. aeruginosa*, which was attenuated by a higher inoculum. The pharmacodynamics of polymyxin B was most closely linked to the AUC/MIC ratio. In conjunction with toxicity data, a dose higher than the standard dose and/or combination therapy may be necessary to suppress *P. aeruginosa* resistance in immunocompromised hosts.

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